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A Secreted BMP Antagonist, Cer1, Fine Tunes the Spatial Organization of the Ureteric Bud Tree during Mouse Kidney Development

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Abstract

The epithelial ureteric bud is critical for mammalian kidney development as it generates the ureter and the collecting duct system that induces nephrogenesis in discrete locations in the kidney mesenchyme during its emergence. We show that a secreted Bmp antagonist Cerberus homologue (Cer1) fine tunes the organization of the ureteric tree during organogenesis in the mouse embryo. Both enhanced ureteric expression of Cer1 and Cer1 knock out enlarge kidney size, and these changes are associated with an altered three-dimensional structure of the ureteric tree as revealed by optical projection tomography. Enhanced Cer1 expression changes the ureteric bud branching programme so that more trifid and lateral branches rather than bifid ones develop, as seen in time-lapse organ culture. These changes may be the reasons for the modified spatial arrangement of the ureteric tree in the kidneys of Cer1+ embryos. Cer1 gain of function is associated with moderately elevated expression of Gdnf and Wnt11, which is also induced in the case of Cer1 deficiency, where Bmp4 expression is reduced, indicating the dependence of Bmp expression on Cer1. Cer1 binds at least Bmp2/4 and antagonizes Bmp signalling in cell culture. In line with this, supplementation of Bmp4 restored the ureteric bud tip number, which was reduced by Cer1− to bring it closer to the normal, consistent with models suggesting that Bmp signalling inhibits ureteric bud development. Genetic reduction of Wnt11 inhibited the Cer1-stimulated kidney development, but Cer1 did not influence Wnt11 signalling in cell culture, although it did inhibit the Wnt3a-induced canonical Top Flash reporter to some extent. We conclude that Cer1 fine tunes the spatial organization of the ureteric tree by coordinating the activities of the growth-promoting ureteric bud signals Gdnf and Wnt11 via Bmp-mediated antagonism and to some degree via the canonical Wnt signalling involved in branching.

Introduction

Kidney development is initiated when a morphologically distinguishable ureteric bud forms and invades the predetermined metanephric mesenchyme and goes on to induce nephrogenesis [1–3]. While generating the ureter and the collecting duct system with a defined pattern, the branches of the ureteric tree specify the locations where nephrogenesis is to be initiated. Each of the ureteric branches induces nephrogenesis via Wnt9b signalling, after which Wnt4 initiates mesenchyme-to-epithelium transition to generate a segmented nephron [4–7].

In recent years critical signalling networks have been identified that are associated with the initiation of ureteric bud formation. An embryonic kidney mesenchyme-expressed Glial cell line-derived neurotrophic factor (Gdnf) and its receptors are important initiators, and several upstream and downstream components have been identified that contribute to the patterning and timing of ureteric bud development via Gdnf control [3,8–15]. Fgf antagonism by Sprouty controls the sensitivity of the ureteric bud to Gdnf [16,17] via an Fgf10-dependent mechanism [18], and signals from the Bmp family are also involved in the initiation of ureteric bud development [19,20], although two of them, Bmp2
and Bmp4, are considered to act as inhibitors of the process [21–24].

Much less is known about the mechanisms that control the later steps in ureteric bud branching, i.e., the establishment of the complex spatial organization of the ureteric tree, which represents the future collecting duct system. Gdnf/Ret appears to have some role, and this together with Wnt11 exerts a positive feedback effect on early ureteric bud development [25].

The mode of action of the Bmps is regulated by a panel of extracellular anti-Bmp and pro-Bmp activity factors such as Crossveinless2, representing a Bmp agonist in the developing kidney [26]. The Cerberus/Dan family forms one group of secreted Bmp antagonists that includes the mCerberus 1 homologue (Cer1), Prdc, Dan, Dm (Gremlin), Sost/Ectodin/Wise/USAG1 [27–30] and Dte proteins [31–34]. Gremlin advances early ureteric bud formation by antagonising Bmp4/Bmp7 signalling [35–37], while USAG1 may serve as a Bmp7 antagonist in the more advanced kidney [38]. Cerberus encodes a Spemann’s organizer signal and binds and inhibits Bmp, Wnt and Nodal signalling [39,40].

Dissection and culture of the embryonic kidney

The kidneys were isolated at E11.5 (45–47 somites), incubated for one minute in 3% pancreatin/trypsin (Gibco-BRL) in Tyrode’s solution [47] and the mesenchyme and ureteric bud separated out mechanically and subjected to RT-PCR. The kidneys were cultured in the presence of Gdnf (100 ng/ml, R&D systems) [16,47] or 100 ng/ml [21] of Bmp4 (R&D system), fixed and processed for immunostaining according to Chi et al. [16,47]. The culture times were as indicated in the Results section.

RT-PCR

The embryonic kidneys were dissected in ice-cold phosphate-buffered saline (PBS), pH 7.4, and total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen). cDNA was synthesized using the RevertAid™ first-strand cDNA synthesis kit (Fermentas) and the RT-PCR was performed according to Chi et al. [47]. The primers, the conditions for PCR and the expected fragments are indicated in Table S2.

Immunofluorescence

Rabbit polyclonal Pax2 antibody (BioSite), anti-cytokeratin endoantibody, Troma-I, recognizing a ureteric bud-specific component in the early kidney (Developmental Studies Hybridoma Bank, USA), and anti-pSMAD antibodies binding to SMADs 1, 5 and 8 (Cell Signalling Technology) were used. Alexa 488 anti-rabbit IgG and Alexa 546 anti-rat IgG served as the secondary antibodies (Molecular Probes, Invitrogen Detection Technologies). Images were captured with an Olympus Fv1000 confocal laser scanning microscope and an Olympus BX51WI upright microscope connected to a Hamamatsu ORCA-ER digital camera. The CellM, Adobe, Photoshop CS and CorelDRAW 12 programs were used for image processing. A minimum of five samples were analysed.

Time-lapse imaging

Dissected E11.5 kidney rudiments were placed on permeable polyester membranes of porc size 0.4 μm and cultured in DMEM (Gibco 41965) supplemented with 10% FBS and 1% penicillin/streptomycin on Transwell plates (Costar 3450) in a microscope stage incubator (OkoLab). The temperature was set to 37°C and the level of carbon dioxide to 5% with the TControl Basic 2.3. Program (OkoLab). The samples were photographed every 20 min until 120 hrs with an Olympus IX81 microscope and an Olympus CC-12 digital camera supported by the Olympus CellF pro program.

Histology, in situ hybridization and detection of the cells in the S-phase of the cell cycle

Certain embryonic kidneys were fixed in Bouin’s solution, sectioned and photographed with a Leica CD 100 digital or

**Methods**

**Ethics Statement**

All genetic studies involving mice were performed in strict accordance with the Finnish law, act 62/2006 on Animal Experimentation following the approval by Finnish National Animal Experiment Board, ELLA. The board donated the authority for the local institutional ethics committee to approve the study with an ID 14/2009 (valid until 31-12-2011) since only ex vivo samples from the generated transgenic mouse lines were used. All the animal experiments here in were classified as grade zero, which imply minimal suffering of mice. The 3R principles were strictly implemented as required by the Finnish laws governing experimental studies involving animals. The animal care and other procedures in this work were also in accordance with the use of laboratory animals and European Union requirements (ETS 123 and Directive 86/609/EEC).

**Mouse lines**

A 4.3kb Pax2 promoter fragment was used to target Mus musculus Cerberus 1 homologue (Xenopus laevis) (Cer1, NM_009687) gene expression [16,43] to the ureteric bud. An IRESGFP cDNA was inserted downstream of the Cer1 gene (Figure 1E) as verified by PCR of DNA samples derived from ear clips. An expected 1000 bp fragment detected in the three transmitting transgenic mouse lines was named Cer1 and selected for closer study (Table S1). The Cer1 transgene positive males were crossed with wild-type C57BL6 females to obtain embryos or mice for this purpose.

The HoxB7/Ce and floxed Rosa26 yellow fluorescent protein (YFP) mouse lines have been described previously [10,46,47], while the Cer1; Wnt11+/− and Cer1; HoxB7/CeYFP+ mouse lines were generated by crossing the Cer1;Wnt11+/− and HoxB7/CeYFP+/− lines. The Cer1 knockout mouse has been described earlier [43].
Olympus DP 500 camera, after which the images were processed with the Adobe Photoshop and Corel Draw programs. Glomeruli were counted according to Bertram et al. [48]. The total kidney volume was calculated as the sum of the volumes of the sections. The OPT and Metamorph programs were also used to estimate the volumes. The surface area was counted with the ImageJ program [http://rsb.info.nih.gov/ij/] [49] and OPT [50–52]. In situ hybridization was performed according to Kispert et al. [53] and Chi et al. [16]. A minimum of five samples were analysed for each gene. The probes for the Gdnf, Ret and Wnt11 genes were obtained as gifts. The number of cells in the S-phase of the cell cycle was evaluated with a kit (Amersham Bioscience, UK) from a minimum of four kidneys for each developmental stage.

Optical projection tomography (OPT) and the degree of ureteric bud branching

A minimum of eight embryonic E15.5 kidneys of each genotype were fixed as whole mounts in methanol and processed for optical projection tomography (OPT) [50–52]. Prior to OPT some kidneys of the Cer1 knockout embryos were subjected to whole mount in situ hybridization analysis to reveal Wnt11 expression. The separated kidney rudiments were placed into TBST with 10%
medium of the normal L1 cells served as the control. After the culture the cells were lysed with Cell Culture Lysis Reagent (Promega) and the Luciferase Assay System (Promega) was used to estimate the influence of Cer1 on Bmp/Wnt signalling as measured with the Victor3V Multilabel Counter (Perkin Elmer). β-galactosidase activity was monitored in 25 mM MOPS, 100 mM NaCl and 10 mM MgCl2 and the substrate. The monoclonal anti-mouse Cerberus 1 antibody (MAB1986) was from R&D systems, and Western blotting was performed according to Raloi et al. [56].

Results
Expression of Cerberus/DAN family members in the embryonic kidney

Cerberus/Dan family members include the mCerberus 1 homologue (Cer1), Prlc, Dan, Drm (Gremlin) and the Dte proteins. To gain an insight into their potential role in kidney organogenesis, we ascertained whether they are expressed in the embryonic kidney. PCR and in situ hybridization studies with isolated ureteric buds (U), the metanephric mesenchyme [KM] and whole kidneys (K) revealed that besides Dmm [36], Cer1, Dan and Prlc are expressed in the ureteric bud and kidney mesenchyme at E11.5 (Figure S1A, C), at E12.5 and E15.5 (Figure S1A, D). It should be noted that Cer1 expression at E11.5 is weaker in the ureteric bud (U) than in the kidney mesenchyme (KM, Figure S1A) and that expression takes place throughout the epithelium and mesenchyme (Figure S1C, D). The presence of Cerberus/Dan gene family members suggested a role for these factors in kidney organogenesis, and out of these members we focused our attention on Cer1.

is involved in kidney development: Evidence from gain and loss of function studies

Since we found that the ureteric bud cells expressed less Cer1 than the metanephric mesenchymal cells (Figure S1A), we speculated that this may be relevant for the establishment of gradients of growth factors bound by Cer1, such as the Wnts and Bmps [40,43] involved in ureteric bud development. We used a ureteric bud-specific 5′ promoter element from the Pax2 gene (Figure S1E) [16,45] to direct Cer1 and an IRES-eGFP reporter gene to the Wolffian duct-derived ureteric bud to increase the level of Cer1 expression.

We generated three transgenic mouse lines, all named Cer1, and these gave similar results. The Pax2 promoter directed Cer1 and eGFP expression in the ureteric bud and increased Cer1 expression to a level closer to that seen in the mesenchymal cells (Figure S1, compare B with A). No eGFP expression was detected in wild-type kidneys at any stage (Figure S1F-H); eGFP expression was intense in the Wollfian duct at E10.5 and in the ureteric bud at E11.5 (Figure S1I, arrow, and data not shown), while at a still later developmental stage, E15.5, eGFP was localized to the ureteric tree (Figure S1J). Expression persisted in the kidney at birth (Figure S1K).

Elevation of Cer1 expression led to a notable phenotype in the kidney, since enlargement of the Cer1+ kidneys was observed in 18 out of the 24 cases of newborn Cer1 mice analysed relative to their wild-type controls (Figure 1, compare B with A). Morphometric studies of a panel of Cer1+ kidneys (see Figure 1H) revealed that the volume of the Cer1 kidneys was around 20% greater, their weight 28% greater and the number of glomeruli 21% greater than in their wild-type littermates (Figure 1H, Table 1). It was significant that a second ureteric bud (U1/U2) had developed in 2 out of the 36 Cer1 kidneys at E12.5 (Figure 1, compare J, F with I, E, arrows). The greater size of the Cer1 kidneys persisted in the adults (Figure S2, compare B, D with A, C), as depicted
As was the case with the kidneys of \( \text{Cer1}^{+} \) newborn mice, the kidneys of the newborn \( \text{Cer1} \) knockout mice were also around 12% greater in size than their controls (data not shown).

The above \( \text{Cer1} \)-promoted kidney development could have arisen for a variety of reasons, such as changes in cell or tissue size or in cell shape and/or stimulated cell proliferation, which could be viewed collectively as changes in ureteric bud branching during organogenesis. Counting of the cells in the S-phase revealed around 30% more BrdU-positive cells in the \( \text{Cer1} \) kidneys than in the controls at E15.5 (Figure 1, compare N with M), as depicted quantitatively in Figure 1O. We conclude that both enhanced \( \text{Cer1} \) expression and \( \text{Cer1} \) deficiency increase kidney size, indicating a function for \( \text{Cer1} \).

Enhanced \( \text{Cer1} \) expression in the ureteric bud changes the mode of branching

Since Bmp signalling is implicated in ureteric bud development [20,57] and as \( \text{Cer1} \) binds Bmps and Wnts in other systems [39], we speculated that \( \text{Cer1} \) may influence kidney size by exercising control over ureteric bud development. We studied this aspect by crossing the \( \text{HoxB7Cre} \) and floxed \( \text{Rosa26} \) yellow fluorescent protein (\( \text{R26RYFP} \)) mice with those that contained the \( \text{Cer1} \) transgene [10,45,46] and monitored the generation of ureteric tips in vivo and in embryonic kidney cultures.

Counting of the ureteric bud tips in the \( \text{HoxB7Cre}^{+} \)/\( \text{R26RYFP} \) and the \( \text{HoxB7Cre}^{+} \)/\( \text{R26RYFP};\text{Cer1}^{+} \) kidneys at E15.5 showed that these were around 10% greater in number in the \( \text{Cer1}^{+} \) kidney than in the corresponding wild-type organs (Figure 2, compare D with C), as depicted quantitatively in Figure 2E noted to some extent already at E12.5 (Figure 2, A,B,E). Given this, we also analysed the pattern of ureteric bud branching by cultivating the E11.5 kidneys. This set-up is free of any possible \( \text{Cer1} \)-induced systemic kidney-affecting factors, since the \( \text{Pax2} \) promoter is not exclusively targeting the kidney [45]. Examination of embryonic kidneys cultured up to 96 hrs indicated that \( \text{Cer1} \) had given rise to a moderate enhancement of ureteric bud tip formation relative to the controls at each time point analysed.

### Table 1. \( \text{Cer1} \)-induced changes in kidney volume, weight and number of glomeruli in different genetic backgrounds.

<table>
<thead>
<tr>
<th>Genotype (NB)</th>
<th><strong>Analyzed</strong></th>
<th>Volume (mm(^3)) *</th>
<th>Weight (mg)</th>
<th><strong>Glomeruli</strong> **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>10</td>
<td>5.29±0.75</td>
<td>6.82±0.91</td>
<td>1475±526</td>
</tr>
<tr>
<td>( \text{Cer1} )</td>
<td>8</td>
<td>6.34±0.82 (↑ 20%)</td>
<td>8.71±0.62 (↑ 28%)</td>
<td>1782±782 (↑ 21%)</td>
</tr>
<tr>
<td>( \text{Cer1}^{+}/- )</td>
<td>8</td>
<td>6.03±0.78</td>
<td>8.12±0.76</td>
<td>1681±884</td>
</tr>
<tr>
<td>( \text{Cer1}^{+}/- )</td>
<td>6</td>
<td>4.82±0.83</td>
<td>6.62±0.58</td>
<td>1494±435</td>
</tr>
<tr>
<td>( \text{Wnt11}^{+/-} )</td>
<td>8</td>
<td>5.05±0.95</td>
<td>7.16±0.64</td>
<td>1405±399</td>
</tr>
<tr>
<td>( \text{Wnt11}^{+/-} )</td>
<td>6</td>
<td>3.43±1.76</td>
<td>5.07±1.29</td>
<td>1178±126</td>
</tr>
</tbody>
</table>

*The total volume of a panel of kidneys from newborn mice was calculated by obtaining the sum of the volumes of the histological sections.

**The number of glomeruli was estimated according to Bertram et al. [48]

![Figure 2. Targeted ureteric bud \( \text{Cer1} \) expression increases the number of ureteric bud tips. A-D. Ureteric buds with yellow fluorescent protein (YFP) expression that was activated from the \( \text{Rosa26} \) locus with \( \text{HoxB7Cre} \). E) Counts of ureteric tips in freshly separated embryonic kidneys indicate that \( \text{Cer1} \) gain of function had led to a moderate elevation in the number of ureteric bud tips seen at E15.5. Time-lapse micrographs of wildtype embryonic kidneys isolated from E11.5 embryos cultured for up to 96 hrs (F-I) and those of embryos overexpressing \( \text{Cer1} \) (J-M). N) Depicts the ureteric tip numbers in wild-type and \( \text{Cer1} \) embryonic kidneys. \( \text{Cer1} \), transgenic kidney overexpressing the \( \text{Cer1} \) gene; Wt, wild-type. Scale bar, 100 μm.

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(Figure 2, compare J-M with F-I). Cer1+ had also stimulated the formation of some lateral side branches and trifurcations of the ureteric bud tip region (Figure 2K, arrows), as depicted quantitatively in Figure 2N.

Closer analysis of the lengths of the early ureteric buds in still images extracted from the time-lapse movies of cultured E11.5 wild-type and Cer1+ kidneys revealed that Cer1 had a notable positive effect on the length of the new branches of the ureteric bud by comparison with the controls when studied at 24hrs and 48 hrs of culture up to the 5th generation (Figure S3). These alterations in ureteric branching are apparently the reasons for the characteristic changes in the overall generation and appearance of the ureteric bud branching pattern, as seen in the time-lapse movies (Movie S1) and still images from them (Figure S4, compare B, D, F, H, J with A, C, E, G, I). Consistent with the in vivo situation, analysis of the movies and still images indicated that Cer1 expression had a tendency to reduce the formation of the bifid type of branches and promote the formation of proportionally more trifid and lateral-type side-branches relative to the total number of branches as compared with the control cultures (Table 2; Movie S1, Figure S4, arrowheads).

To obtain a better view of the overall organization of the ureteric tree generated from the bud, the presumptive collecting duct and the changes that were caused by Cer1 gain or loss of function in vivo. We stained the whole collecting duct system with anti-cytokeratin 19 antibodies as whole mounts and subjected the kidneys at E15.5 to optical projection tomography (OPT) [50–52]. Preliminary results suggested that the distance of the first ureteric bud branch points that were closest to the pelvis from the mean centre was reduced by Cer1+ as compared with the controls (Figure S5). In more detailed studies it became evident that Cer1 had already enhanced the number of ureteric tips and the size of the pelvis by E15.5 (Figure 3, A-H, compare N to M; Movie S2). Calculation of the OPT data with the Imaris program revealed a number of indicative parameters for the ureteric tree, enabling us to generate OPT-derived values for the ureteric bud (Table 3). The Cer1+ kidneys had 25% more tips and 29% more branch points then the controls (Table 3), while the Cer1-deficient kidneys were also characterized by considerable changes in the OPT-derived values for the ureteric bud (Table 3). The Cer1+ kidneys at E15.5 of development had a 30% greater surface area, 26% greater length, 30% higher volume and a 20% increase in the average distance between the ureteric tips at the developing cortex, as identified by counting the distances between Wnt11-positive tips in 3D from the OPT data (Figure 3 I-L, and O-P; Table 3) by comparison with the littermate controls (Cer1+/-). Hence both Cer1+ and Cer1 deficiency lead to changes in several parameters indicative of ureteric tree organization and support the conclusion that Cer1 plays a role in fine tuning the spatial organization of the ureteric tree.

Changes in Cer1 function lead to a tendency for altered expression of Gdnf/Wnt11, which form a signalling loop exercising positive control over ureteric bud development

To address the questions of when and how enhanced Cer1 expression starts to promote ureteric bud development, we prepared embryonic kidneys at E11.0-E11.5, the stage at which the bud has just formed and has invaded the metanephric mesenchyme. As judged by whole mount in situ hybridization, expression of the Gdnf gene appeared to be somewhat enhanced in the mesenchyme due to Cer1 expression relative to the controls (Figure 4, compare B with A, arrows). The expression of the Gdnf receptor Ret indicated that Cer1 expression had already enhanced ureteric bud development at E11.5, while the first branch had not yet been initiated in the control embryos matched with them on the basis of somite numbers (Figure 4, compare D with C, arrows, see Movie S1). Wnt11, a signal that promotes early ureteric bud development [25,58] was maintained in the ureteric bud tips, which were slightly more numerous, supporting the conclusion that Cer1 had also accelerated ureteric bud tip development at 12.5 (Figure 4, compare F with E).

It is known that the Gdnf/Ret and Wnt11 pathways advance ureteric bud development synergistically [25]. Real-time PCR analysis of the kidneys of wild-type and Cer1+ embryos suggested that the expression of both Gdnf and Wnt11 had a tendency to be up-regulated on account of Cer1 expression as compared with controls (Figure 4 G, H). To test further the potential involvement of Gdnf in Cer1-mediated control, we supplemented the cultures of Cer1+ embryonic kidneys with Gdnf. The Cer1+ kidneys appeared to be more sensitive to exogenous Gdnf than their wild-type controls, since Gdnf in a concentration of 100ng/ml [16,59] induced more pronounced supernumerary Wolffian duct-derived epithelial bud formation in the Cer1+ kidneys than in the wild-type controls. The ectopic epithelial buds had also induced more foci of mesenchymal cells expressing Pax2 in the Cer1+ embryonic kidneys than in the controls (Figure 5, compare E with D and A, arrows, arrowheads), as also illustrated in the ureteric tip counts for the cultured samples (Figure 5H). We interpret the results collectively as supporting the conclusion that Cer1 influences Gdnf and Wnt11 gene expression in order to stimulate ureteric bud development.

Wnt11 is partially involved in mediating the positive effect of Cer1 on kidney development

If Wnt11 were involved in Cer1 control, a genetic reduction in Wnt11 in a heterozygous Wnt11+/-background might inhibit the influence of Cer1 in promoting ureteric bud development and thus kidney size. We tested the significance of the slightly elevated Wnt11 expression (Figure 4H) for Cer1-promoted ureteric bud development by means of Wnt11-deficient mice [24]. The volume,

Table 2. Cer1 gain of function reduces the proportion (%) of bifid-type ureteric bud branches and increases the proportions of the trifid and lateral types.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Genotype</th>
<th>Type of branching**</th>
<th>Bifid</th>
<th>Trifid</th>
<th>Lateral</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Wt</td>
<td></td>
<td>57%</td>
<td>29%</td>
<td>14%</td>
<td>100%</td>
</tr>
<tr>
<td>Cer1</td>
<td></td>
<td></td>
<td>45%</td>
<td>33%</td>
<td>22%</td>
<td>100%</td>
</tr>
<tr>
<td>48</td>
<td>Wt</td>
<td></td>
<td>71%</td>
<td>17%</td>
<td>12%</td>
<td>100%</td>
</tr>
<tr>
<td>Cer1</td>
<td></td>
<td></td>
<td>63%</td>
<td>22%</td>
<td>15%</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Eight cultured E11.5 kidneys of each genotype were analyzed.
**Modes of ureteric branching estimated according to Watanabe and Costantini [83].

doi:10.1371/journal.pone.0027676.t002
Figure 3. Changes in morphometric parameters of the ureteric tree in Cer1 gain and loss of function situations. Kidneys were prepared from wild-type (Wt) and Cer1 mutant embryos at E15.5 and subjected to OPT analysis to calculate several parameters characteristic of the ureteric tree. The tree was identified in the kidneys stained as whole mounts with the Troma-I antibody, which recognises the cytokeratin antigen expressed by cells of the ureteric tree (A, C, E, G). The surface of the ureteric tree in Wt (B), Cer1+ (D), Cer1+- (F) and Cer1-/ (H) situations highlights alterations in the overall pattern. Wnt11 transcripts are localized to the ureteric tips in the normal kidney at E15.5 (I) and in Cer1 knockout, but Wnt11 expression is elevated in response to Cer1 deficiency (J). The Wnt11 expression pattern was used to count the exact numbers of cortical ureteric tips in Cer1+- (K) and Cer1-/ embryonic kidneys (L) and the distances between them (black lines in K and L). Number of ureteric tips (M) and the width of the kidney pelvis (N) in wild-type (in green) and Cer1+ (in red) embryonic kidneys. Diagrams depicting the average length of the ureteric tree (O) and the distance between its tips (P) in Cer1+- and Cer1-/ embryonic kidneys. Cer1, transgenic kidney overexpressing the Cer1 gene; Wt, wild type. Scale bar, 100 μm.
doi:10.1371/journal.pone.0027676.g003
weight and number of glomeruli in the kidneys of the Cer1; Wnt11+/− mice was indeed smaller than in the Cer1+ individuals (Figure 1H, compare 1C to B, Table 1), but Cer1 had still promoted these parameters in the Wnt11-deficient kidney, since the values were higher in the Cer1; Wnt11−/− mice than in the Wnt11−/− mice (Figure 1, compare D to C, H; Table 1). We conclude that Wnt11 is involved in mediating the influence of Cer1 on kidney development.

Cer1 down-regulates the expression of Bmp4, encoding an inhibitor of ureteric bud development, and binds Bmp2/4 but not Gdnf.

Of the Bmps, it is mainly Bmp2 and Bmp4 that are considered to be inhibitors of ureteric bud development [19–24]. In contrast to the expression of the Gdnf and Wnt11 genes, which showed a tendency for induced expression in response to Cer1 gain of function, Bmp4 expression tended to be reduced by Cer1 at all the stages analysed (Figure 4). Like Bmp4 expression, that of the Gremelin (Grem) gene, which encodes another Bmp4/7 antagonist and is involved in the initiation of kidney development [36,37], demonstrated a tendency to be reduced in embryonic kidneys expressing Cer1 as compared with controls (Figure 4).

When we subjected the Cer1+ embryonic kidneys to organ culture, the ureteric bud in some of them was found to have become split in two at the stalk region. These ectopic ureteric bud branches had induced the formation of Pax2+ cells adjacent to the epithelial buds, indicative of the early steps in tubule induction (Figure 5C, in green, arrow). We consider the formation of a double ureteric bud

**Table 3.** OPT image analysis-derived values for the ureteric tree in the Wt, Cer1+, Cer1+/− or Cer1−/− genotypes at E15.5.

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>Cer1+</th>
<th>%</th>
<th>Cer1+/−</th>
<th>%</th>
<th>Cer1−/−</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tips #</td>
<td>422±55</td>
<td>528±51</td>
<td>25%</td>
<td>438±45</td>
<td>10%</td>
<td>480±69</td>
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<td>Branch points #</td>
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<td>257±23</td>
<td>29%</td>
<td>209±26</td>
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<td>234±33</td>
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<td>Average distance between branch points (μm)</td>
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<td>76.39</td>
<td>1%</td>
<td>72.5±36</td>
<td>13%</td>
<td>82.5±42</td>
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<td>Surface area (mm²²)</td>
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<td>265.26</td>
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<td>20%</td>
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<td>Pelvis width (μm)</td>
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doi:10.1371/journal.pone.0027676.t003

**Figure 4.** Cer1 gain of function leads to reduced Bmp4 expression but induced Gdnf and Wnt11 expression. A, B) Localization of Gdnf gene expression (arrows) in the mesenchyme of a wild-type (A) and Cer1+ embryonic kidney (B). The dotted lines in (A) and (B) depict the border of the ureteric bud. C, D) Ret expression reveals that the ureteric bud is advanced in development at E11.5 in the case of Cer1+ as compared with a stage-matched control (arrows in C and D). E-F) Wnt11 expression reveals that the Cer1 gain of function has promoted ureteric bud development at E12.5 as compared with the degree of tip development in the control. G-J) Analysis of changes in Gdnf, Wnt11, Bmp4 and Gremelin (Grem) expression brought about by qPCR point to trend for average reductions of 24% in Gdnf (G), 14% in Wnt11 (H), 21% in Bmp4 (I) and 15% in Grem (J) relative to the controls upon Cer1 gain of function. Cer1+, transgenic kidney overexpressing the Cer1 gene; Wt, wild-type. Scale bar, 50 μm.
doi:10.1371/journal.pone.0027676.g004
as the likely reason for the development of a second kidney noted in certain Cer1 mutants (see Figure 1G, K (K1 and K2, arrows)).

We went on to test directly whether Bmp4 would regulate Gdnf and Wnt11 expression in the early embryonic kidney under normal conditions, since we noted a correlation between the tendencies for a reduction in Bmp4 expression and an increase in that of Gdnf and Wnt11. Supplementation of the culture medium with 50 ng/ml Bmp4 [23] reduced Gdnf expression by 28% and Wnt11 by 20% as compared with controls treated with bovine serum albumin (BSA) (Figure 5I). The same amount of Bmp4 in cultures of E11.5 Cer1 embryonic kidneys reduced Cer1-induced ureteric bud branching and brought the number of ureteric tips closer to that found in the cultured wild-type embryonic kidneys (Figure 5, compare B with G), as depicted quantitatively in Figure 5H. Consistent with earlier data [57], supplementing the Bmp4 in the embryonic kidney cultures inhibited the number of tips in the wild-type embryonic kidneys to some extent relative to non-treated wild-type controls (Figure 5F).

Cer1 binds Bmp2 and Bmp4 but not Gdnf, and antagonizes Bmp signalling

Given the suggestion that Wnt11, Gdnf and Bmp4 are involved in mediating Cer1-stimulated ureteric bud branch development, we speculated that Cer1 would bind directly to Bmps and in that way lower their presence in the embryonic kidney in order to stimulate branching, as would be consistent with the earlier model of Bmp4 action serving as an inhibitor [21]. To address this aspect, we analysed the capacity of the mCer1 protein to bind to Bmp2, Bmp4 and Gdnf and the potential of Cer1 to influence Bmp, Wnt11 and canonical Wnt signalling in cell lines by comparison with the control situation.

BLAcore sensogram analysis revealed that mCer1 bound Bmp2 and Bmp4 but not Gdnf (Figure 6). Moreover, Bmp4 recombinant protein induced Bmp (Snail) reporter gene expression in a dose-dependent manner, so that Bmp4 induced the maximal reporter activity at concentrations of 33 ng/ml and 50 ng/ml (Figure 7A). Having revealed the dose response associated with Bmp4, we used...
different amounts of Bmp4 to test whether the Bmp4 signalling output would depend on its concentration and could shed light on the phenotypes generated by Cer1 gain and loss of function. We used Wnt4 as a means of addressing the potential role of Cer1 in orchestrating not only ureteric bud development but also mesenchymal cell behaviour.

Strikingly, the effect of Bmp4 was clearly dose-dependent, so that lower amounts of Bmp4 inhibited the expression of Wnt4, which is a critical regulator of nephrogenesis [4], whereas higher amounts induced it (Figure 7B). Given these observed Bmp4 thresholds, we went on to determine whether mCer1 would indeed act as an inhibitor of Bmp4 signalling in the kidney. Cer1 inhibited the Bmp reporter induced by Bmp4 recombinant protein and cDNA transfections in a dose-dependent manner both in embryonic kidney-derived mK4 cells and in CHO cells (Figure 7C-E). It is worth noting, however, that the inhibition achieved with Cer1 was weaker than with Noggin in the reporter gene assay (Figure s7, compare F with C-E), which is in line with the notion of Cer1 playing a role as a fine tuner of kidney development.

Given the involvement of Wnt11 in the control of Cer1-induced ureteric bud development, we analysed whether Cer1 would influence Wnt11 signalling. Since no direct Wnt11 reporters are currently available, we assayed the influence of Cer1 in the control of Wnt11 signalling indirectly by assessing the capacity of Wnt11 to inhibit canonical Wnt signalling as analysed by the Top Flash reporter [56]. Hence, if Cer1 influenced Wnt11 signalling, we would expect to see changes in the Wnt11-mediated inhibition of Top Flash reporter expression [56]. However, Cer1 did not influence the efficiency of Wnt11 in inhibiting Wnt3a-induced Top Flash activity, although it had a notable effect in reducing Wnt3a signalling (Figure 7G).

Changes in Cer1 function influence Bmp4 expression and signalling

Our findings suggested that Cer1 controls ureteric bud development by antagonizing the inhibitory effect of Bmps on bud branching, and possibly by affecting the kidney mesenchymal cells directly, since Bmp4 had a dramatic effect on Wnt4 expression in the cell line models. Given these results, we studied further the role of Cer1 in the control of Bmp2, Bmp4 and Wnt4 expression in Cer1 knockout (Cer1-/-) and Cer1+ embryonic kidneys. Cer1 deficiency did indeed reduce Bmp4 expression in comparison to controls, and to some extent also that of Bmp2 and Wnt4, as judged by real-time PCR (Figure S6A-C). Analysis of phospho-Smad (pSmad), which is normally expressed in the ureteric bud-derived collecting duct tree, and its pretubular derivatives (Figure S6D), showed that both a gain in Cer1 function and Cer1 deficiency notably reduced pSmad expression in the embryonic kidney as judged by immunostaining (Figure S6, compare E-F with D), which is consistent with the reduction in Bmp 2/4 ligands in the kidneys of Cer1 mutant newborn mice (Figure 4I and Figure S6A, B).

Discussion

Our data indicate that the secreted Bmp antagonist Cer1 is involved in the spatial organization of the ureteric tree during
kidney development. This conclusion is based on the fact that Cer1 gain of function and Cer1 deficiency both enlarged kidney size and this was associated with changes in the overall appearance of the ureteric tree and in several developmental ureteric bud parameters identified by coupled optical projection tomography (OPT) imaging of the whole collecting duct system. We consider that manipulation of Cer1 causes these phenotypes in part through changes in Cer1-mediated antagonism of Bmp signalling, on the grounds that Cer1 bound Bmp2/4, as analysed by surface plasmon resonance technology, Cer1 inhibited Bmp4 signalling, as judged by changes in the Bmp reporter, both Cer1 gain and loss of function led to reduced Bmp4 expression, and supplementation of Bmp4 in Cer1-expressing embryonic kidneys in organ culture lowered the number of Cer1+-induced ureteric bud tips to bring it closer to the wild-type control value. On top of these findings we also noted that Cer1 is clearly a weaker Bmp antagonist than Noggin, another Bmp antagonist that influences kidney development [60]. Our findings are in line with the mode of action of Bmp2 and Bmp4 as inhibitors of ureteric bud development [21,24,61,62]. Given these points, we consider that Cer1 serves as a secreted factor that partially controls Bmps activity in the coordination of ureteric bud development by fine tuning the branching process.

Consistent with the proposal that Cer1 functions in the developing kidney to control Bmp-mediated functions in the assembling ureteric bud, the bud is responsive to certain Bmps via canonical Smad-mediated signal transduction [19,21,36,63]. Even though a wealth of studies have been performed to define the expression of Bmp ligands during kidney development, the picture is not completely clear. The kidney expresses at least Bmps 2/3/4/5/6/7 in partly overlapping regions, but also in clearly different compartments. Considering the results collectively, however, it is evident that the Bmps are expressed in both the ureteric bud and the mesenchymal cells, including the condensed kidney mesenchyme, the survival of which involves Bmp7 function [19,20,57,64]. It is significant that Bmp2/4 is not initially present in the condensed kidney mesenchymal cells but is up-regulated during nephrogenesis [57]. Thus, besides having a key role in the initiation of kidney development, the expression pattern of the Bmp family members suggests a role later in kidney development, namely in controlling the ureteric bud in the establishment of the complex ureteric tree structure and nephron development. Evidence is available that Bmp4 takes part in specification of the proximal distal identity in ureteric bud development, for example [65].

Since we already found Cer1 expression by E11.5, at the initiation of kidney development in the ureteric bud and kidney mesenchyme, it could in principle influence Bmp function in both of these tissue layers. It was also evident that the level of Cer1 expression was lower in the ureteric bud than in the kidney mesenchyme. We regarded this as an opportunity to address Cer1 function in controlling the activity of certain growth factors such as Wnts and Bmps in order to target the mechanisms behind ureteric bud development. Overexpression of Cer1 in the ureteric bud as induced by the Pax2 promoter led to phenotypes in branching that suggested a role for endogenous Cer1 in ureteric bud development. Detailed analysis of the OPT data revealed that Cer1 gain of function had stimulated values related to the ureteric tree such as the number of tips, the branch points and the total length of the tree at E15.5. When these parameters were identified at the same developmental stage in the Cer1 knockout (Cer1-/-) the changes turned out to be predominantly in a different set of values, as the values for the ureteric tree surface area, volume, length and distance between the tips at the developing cortex were all greater in the Cer1 knockout embryonic kidney than in the controls. However, the number of ureteric tips and the average distance between the branch points, which were altered in response to Cer1 gain of function, were not changed as clearly as was the case in the kidneys of the Cer1 knockout embryos. Hence, even though Cer1 gain and loss of function both enhanced kidney size and demonstrated reduced Bmp4 expression, the OPT-derived data, for example, reveal that the phenotypes of these mutants differ to a certain degree. These differences may reflect the differing genetic makeup of the transgenic mice used in the gain and loss of function models. We summarize these results as supporting the conclusion that the control of Bmp signalling by Cer1-mediated antagonism is relevant to the fine tuning of the action of Bmp function in establishing the specific pattern of the ureteric tree during kidney development.

Besides the changes in Bmp expression in the Cer1 mutant embryonic kidneys, we also found that Cer1 expression in the ureteric bud had a tendency to stimulate Wnt11 and Gdnf gene expression. These data and the findings that genetic reduction of Wnt11 function in heterozygous Wnt11-deficient embryonic kidneys detracted them from Cer1-induced kidney development, that Cer1+ kidneys were also sensitive to the Gdnf signal and that Bmp4 inhibited Gdnf expression in wild-type embryonic kidneys support a role for Cer1 in the control of Gdnf and Wnt11 [21,66]. Wnt11 functions as a ureteric tip bud signal that especially controls formation of the trifurcation type of ureteric bud branching during the early stages of kidney development [25]. Thus the changes in the mode of ureteric bud branching brought about by Cer1 gain and loss of function may be explained in part by the alterations in Gdnf and Wnt11 expression.

It has been established that besides nephrogenesis, ureteric bud development is also regulated by the Wnt signalling pathway [67]. On top of having an effect on Bmp signalling, we noted a
moderate effect of Cer1 on the canonical Wnt signalling, since Cer1 inhibited Wnt3a-induced Wnt Top Flash reporter expression in a cell line model as compared with controls. The mediators of Bmp signalling, the Smad proteins, can interact with the canonical Wnt signal transduction components β-catenin and Lef1 (TCF) [60], and the Bmp receptors Alk3 and β-catenin are also known to cooperate in the embryonic kidney [60]. Moreover, down-regulation of Wnt9b and the associated canonical Wnt signalling in the ureteric tip region appears to be important for the initiation of ureteric bud branching [67]. Cer1 may contribute to the promotion of ureteric bud branching by influencing canonical Wnt signalling in the tip region. Sostdc1, another Cerberus/DAN family member, may point to a possible mode of action. Sostdc1 regulates β-catenin localization [69] and binds Bmp4 and Lrp4 to inhibit β-catenin-mediated Wnt signalling [70]. Recent data indicate that Lrp4 is also critical for ureteric bud development [71]. Hence, at the same time as it stimulates ureteric bud development by promoting Gdnf Wnt11 expression, Cer1 may contribute to new bud formation during epithelial branching by having an inhibitory effect on the β-catenin-mediated canonical Wnt signalling that occurs in the tip region.

Cer1 is expressed in the kidney mesenchymal as well as in the ureteric bud, and could therefore not only act as a reciprocal signal for controlling the ureteric bud but also in that way contribute to mesenchyme behaviour. Given that the Pax2 promoter targets Cer1 to the ureteric bud, this Cer1 could diffuse to the mesenchyme to some extent, but Cer1 knockout will inactivate the Cer1 contribution in both of these tissue compartments. Hence both gain and loss of Cer1 function may also change causes in the kidney mesenchyme. Thus we found an enhanced tendency for Gdnf expression and a higher number of glomerulae in response to Cer1 gain of function, which may point to a role for Cer1 in the kidney mesenchyme. In line with this possibility, we noted a concentration-dependent effect of Bmp4 on the expression of Wnt4, which is normally expressed in mesenchymal pretubular cells and in an embryonic kidney mesenchyme-derived model cell line. Moreover, not only Bmp4 but also Wnt4 expression was down-regulated by Cer1 knockout to some degree, supporting the idea that their expression is regulated by Cer1. Similar regulatory feedback between Bmp and a Bmp antagonist has been noted between Gremlin and Bmp4/7 during limb bud development [36,37,72] and Bmp4 and Noggin during feather and tooth bud development, Gremlin and Bmp4/7 during limb bud development [36,37,72] between Bmp and a Bmp antagonist has been noted between Gremlin and Bmp4/7 during limb bud development [36,37,72].

It is known that Bmp2/4 are not expressed in the early condensed kidney mesenchyme but that expression is up-regulated during nephrogenesis [20,57,75], while Bmp7 serves as a survival condensed kidney mesenchyme but that expression is up-regulated and Sprouties [17,69,74]. and Bmp4 and Noggin during feather and tooth bud development, Gremlin and Bmp4/7 during limb bud development [36,37,72] between Bmp and a Bmp antagonist has been noted between Gremlin and Bmp4/7 during limb bud development [36,37,72].

Supporting Information

Figure S1 Expression of Cerberus/Dan family and the construct used. A, B) mCerberus 1 homolog (Cer1), Dan and Prdc genes are expressed in the ureteric bud (U) and kidney mesenchyme (KM) of E11.5 embryos and whole embryonic kidneys (K) at E12.5 and E15.5, as revealed by RT-PCR. Note that Cer1 expression is elevated in the ureteric bud in the transgenic embryonic kidney (Cer1, star) relative to the wild-type (Wt) at E11.5 (compare B to A). Like Cer1, Prdc expression is elevated due to the gain of function of Cer1 expression. The Dan/ Cerberus genes, Dan and Prdc are also expressed in the developing kidney. C, D) Whole mount in situ hybridization shows that Cer1 is expressed in both the ureteric bud and kidney mesenchyme at E11.75 and E12.5. E, Schematic structure of the construct used to express Cer1 and eGFP in the ureteric bud. F-H) Wild-type kidneys prepared from embryos at the E11.5, E15.5 and newborn (NB) stages. I-K) Pax2 promoter-driven GFP can be detected in the ureteric bud. The arrow in (I) indicates the ureteric bud of the E11.5 embryonic kidney. NB; newborn. Scale bar, 100 μm. (TIF)

Figure S2 Cer1 gain of function has enlarged the kidney. A) The kidney of a five-month-old wild-type mouse. B) A kidney that has expressed Cer1 in the ureteric bud. C, D) Sections from the kidneys shown in (A, B). Counting the volume of the kidney in six similar samples shown in (A and B) indicates that the kidney that had expressed Cer1 is larger in size than the wild-type control kidney (Wt) (E). Bar 500 μm. (TIF)

Figure S3 Cer1 has a positive effect on the length of the early ureteric bud branches. The kidneys were prepared at E11.5 from embryos that had either YFP only or both the YFP and Cer1 genes (see the methods for details). The length of each ureteric bud branch during early stages of kidney development was calculated according to Watanabe and Costantini (2004) [83] analyzed from still images made from the time-lapse movies recorded of the cultured kidneys. Cer1 has stimulated to a certain degree the length of early branches. At 48 hrs of culture the
branches from the 2nd to 5th generations appear measurable longer.

(TIF)

Figure S4 Still images from time lapse movies from YFP+ ureteric buds of wild-type and Cer1+ embryonic kidneys. The still images from cultures of E11.5 kidneys were used to evaluate the influence of Cer1 on the mode of bifurcation and generation of the ureteric bud branches. Note that Cer1 gain of function has promoted ureteric bud development already at 00 hr (compare B to A, arrowhead), bifurcation of the bud at 24 hr time point (compare D to C, arrowheads) and changes in the overall mode of ureteric branching when compared to the pattern of the ureteric three in later stage cultures of HoxB7/Cre;RIPFPKR26 marked ureteric bud (compare the ureteric tree pattern in F,H,J with E,G,I).

(TIF)

Figure S5 Cer1+ has changed distance of first ureteric bud branch points from the mean centre. The embryonic kidneys were prepared at E15.5 from wild-type or Cer1 embryos, stained as whole mounts with anti-cytokeratin antibody and subjected to analysis of the three dimensional structure of the ureteric tree with optical projection tomography. The morphometric analysis reveal that Cer1 expression diminishes n several samples the distance of the first ureteric bud branch points from the mean center or the kidney when compared to the same parameter values the wild-type (Wt) kidney.

(TIF)

Figure S6 Cer1 loss and gain of function influences Bmp expression and signaling. Real-time PCR analysis of total RNA isolated from kidneys of Cer1 heterozygous (+/-) and wild-type (Wt) embryos, stained as whole mounts with anti-cytokeratin antibody and subjected to analysis of the three dimensional structure of the ureteric tree with optical projection tomography. The morphometric analysis reveal that Cer1 expression diminishes the number of samples the distance of the first ureteric bud branch points from the mean center or the kidney when compared to the same parameter values the wild-type (Wt) kidney.

(TIF)

Figure S7 Schematic representation of the potential modes of action of Cer1 in the control of ureteric branching. As a secreted protein, the Cer1 protein binds Bmp4 in the ureteric bud and the kidney mesenchyme but not Gdnf. Bmp2 and Bmp4 have both been implicated as inhibitory signals for ureteric bud branching involving Alk3 receptor in the ureteric bud. Bmp4 signaling normally leads to repression of the expression of Gdnf, which signals via its Rst receptor expressed in the ureteric bud and promotes ureteric bud development via positive feedback signaling with Wnt11. Lower activity of Bmp due to Cer1 mediated inhibition enhances Gdnf expression and this promotes ureteric bud branching by stimulation of the positive signaling loop between Gdnf and Wnt11 promoting ureteric bud development. Cer1 inhibited to a moderate level canonical β-catenin mediated Wnt signaling and this may be relevant in advancing initiation of branching at the tip region. Modulation of Bmp by Cer1 may also influence mesenchyme which seen changes Wnt4 expression controlling nephrogenesis. Depending of the level of Bmp4 and Cer1, Bmp4 either inhibits or induces Wnt4 expression.

(TIF)

Table S1 Primers used to genotype the generated Cer1+ mouse lines.

(DOC)

Table S2 Primers used to analyse changes in gene expression induced by Cer1+.

(DOC)

Movie S1 Cer1 expression changes the overall pattern of ureteric bud branching when compared to controls. The kidney primordial were prepared at E11.5 and subjected to organ culture for 120 hrs. Ureteric was visualized by genetic means by using yellow fluorescent protein that was activated from the floxed Rosa26 locus as a result of HoxB7/Cre recombination. Analysis of the time-lapse recordings reveal that Cer1 gain of function in the ureteric bud shifts the mode of ureteric bud branching from a bifurcation type towards the trifurcation one (see also Figure S4). C, D) Later Cer1 over expressing kidneys have a tendency to develop also lateral side branches not that typical in the control. As a result the overall pattern formation of the ureteric bud of the kidneys that were prepared from the Cer1 kidneys appears different from the control.

(MOV)

Movie S2 Visualization of Cer1-induced changes in development of the ureteric bud tree during kidney organogenesis analysed. The ureteric bud was identified with antibodies against cytokeratin at E15.5 by using optical projection tomography (OPT). A kidney of a wild-type (Wt) embryo on the left and the one expressing Cer1 in the ureteric bud on the right side identifies Cer1 induced changes in the structure of the ureteric tree.

(MOV)

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Author Contributions

Conceived and designed the experiments: AR RPH IS YY SJV. Performed the experiments: LC US AR RPH IS SA KK YL JS AMS JAB JD YY SJV. Analyzed the data: LC US AR RPH IS SA KK YL JS AMS JAB JD YY SJV. Contributed reagents/materials/analysis tools: LC US AR RPH IS SA KK YL JS AMS JAB JD YY SJV. Wrote the paper: LC AR RPH JD YY SJV. Reagents or tissue: LC AR RPH IS AMS JAB YY.

References